STUDIES OF THE COPPER BINDING PROTEIN IN THE SMALL INTESTINAL MEMBRANE OF BLACK AGOUTI MICE

A THESIS

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BY

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For giving me direction, will power and support in every way possible, I would like to give thanks to my advisor, Dr. Cyril L. Moore, my family, Beverly, my dear friends, and above all my God; for without them all of this endeavor would not have been possible.

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CHAPTER I

INTRODUCTION

Copper is an essential trace element in normal mammalian metabolism. It is a cofactor of enzymes involved in the formation of structural proteins, biogenic amines (neurotransmitters), pigmentation and electron transport. In order for copper to perform its required roles it must first be accessible to the cells or tissues. Normally, nutritional copper is absorbed by the stomach and the small intestine, the duodenal segment in particular (Bush et al., 1956; Van Campen and Mitchell, 1965). The mechanism(s) of absorption is/are not fully understood. Three mechanisms have been proposed: (a) passive or active diffusion (Mills, 1956; Marceau et al., 1970); (b) an energy dependent transport involving a copper amino acid complex absorbed by the mucosa (Crampton et al., 1965; Kirchgessner et al., 1970); and (c) an endocytosis, energy requiring uptake (Sheedlo and Beck, 1981). A cytoplasmic metalloprotein then binds copper (Starcher, 1969; Evans and Cornatzer, 1970; Evans and LeBlanc, 1976; Johnson and Evans, 1980; Prins and Van den Hamer, 1981). This metalloprotein may be responsible for the transport of copper to the portal blood (Johnson and Evans, 1980) where it is then bound by plasma albumin and ceruloplasmin (Evans and Cornatzer, 1970).

The brindle mouse strain (Mo^{br}) is an X-linked recessive mutant with a defect in copper transport. Metabolic disorders are

associated with the resulting copper deficiency (Hunt, 1974). These mice arose spontaneously from an inbred strain of Mus musculus (+/+)(Fraser et al., 1953). The mottled mutant (Mo/+) is in many aspects homologous to Menkes' syndrome in humans (Danks et al., 1973; Hunt, 1974). Therefore, a comparative study of the intestinal uptake and transport of copper in Mo^{br} and in normal mice is important to the enhancement of understanding the defects in Menkes' syndrome.

Many investigators have presented data concerning metalloproteins (Starcher, 1969; Evans and Cornatzer, 1970; Evans and LeBlanc, 1976; Johnson and Evans, 1980; Prins and Van den Hamer, 1981) from cytosolic fractions, but the possibility of a membrane protein involved in copper transport seems to have been ignored for too long. James Everett (1981) isolated a copper binding protein from the membrane fraction of small intestinal mucosal cells. The copper binding protein (or copper transport protein) exhibited on-off (reversible) copper binding kinetics which should be characteristic of a transport protein. Cadmium and zinc were shown to be inhibitors of the binding of copper to this protein. These findings are in agreement with the known effects of cadmium and zinc on the absorption of copper from the diet.

Parental copper feeding of the Mo^{br} diseased male up to day 20-26 postnatally, resulted in the appearance of an identifiable copper transport protein, the absence of which persisted without the copper therapy (Everett, 1981).

The purpose of our investigation is to examine the copper induction of this copper binding protein of the small bowel of the mottled mutant mouse, and the copper binding protein of the normal Black Agouti mouse as a function of age and development. It is our hope that knowledge gained will contribute significantly to greater understanding of intestinal copper absorption and transport to the portal blood.

CHAPTER II

REVIEW OF LITERATURE

Menkes' syndrome, also called Menkes' Kinky Hair Disease (Bray, 1965), Trichopoliodystrophy (TPD)(French et al., 1972), and Menkes' steely Hair (Danks et al., 1973), was first reported in 1962 by John H. Menkes and colleagues. The report was based on the medical cases of five boys from the same family and described as a sex-linked recessive disorder characterized by retardation of growth, abnormally pigmented hair showing pili torti, focal cerebral and cerebellar degeneration, and death of the patient by three and a half years of age. Biochemical studies consistently indicated high levels of plasma glutamic acid, which was speculated to be due to a possible genetic defect of the enzyme, glutamate dehydrogenase, a defect resembling those caused by mercury poisoning. This so-called "new" clinical syndrome was suggested to be caused by a metabolic defect which at that point had not been determined.

Ten years later, Danks et al. (1972b) suggested that Menkes' syndrome was not as rare an occurrence as originally thought. According to case studies done in Melbourne, Australia, there seemed to be a frequency of approximately one in 35,000 live births. These studies also showed that hypothermia was an important clinical feature which had been previously overlooked. With the aid of

arteriography and microscopic examinations, structural abnormalities of arteries were shown and were considered to be the cause of poor vascularization of the brain. Disruption, fragmentation and reduplication of the internal elastic lamina was observed as well as thickening of the intima. Abnormal skeletal changes which resembled scurvy in some ways were found in many patients. Primary cultures of fibroblastic cells from Menkes' patients of the Australian studies, showed metachromasia which was not present in subcultures. A defect in cytochrome oxidase was also noted in these case studies. Similar changes have been indicated in genetic defects in connective tissue or in nutritional copper deficiency of animals (Marston, 1952; O'Dell et al., 1961; Gillespie, 1964; Danks et al., 1972b; Carnes; 1971). Several lines of investigation led to the discovery of copper deficiency in Menkes' syndrome.

1. Gillespie (1964) observed that in copper deficient sheep, there was a paucity of disulfide bonds in the polypeptide chains of wool keratin. This pointed to the similarity between the wool and the hair of Menkes' patients.

2. It was known that copper was required for the activity of lysyl oxidase which catalyzed the oxidation of lysine, which is necessary for the cross linkages in elastin and collagen (Carnes, 1971). This is related to the blood vessel disease associated with Menkes'.

3. The activity of cytochrome oxidase is known to require copper as one of the prosthetic groups of the enzyme. This was related to the hypothermia described by French et al. (1972).

4. Bone development requires a copper-dependent ascorbic acid oxidase (Al-Rashid and Spangler, 1971) found to be deficient in Menkes' patients. Skeletal abnormalities of the patients could be a consequence of this deficiency.

5. The pignemtation (melanization) of hair requires the copper-dependent enzyme, tyrosinase (Lerner et al., 1950).

There is, however, an unclear role of copper in hematopoiesis as judged by the normal levels in erythrocytes and the lack of anemia in the Menkes' disease (Danks et al., 1974). French and coworkers noted, however, a degree of hemolytic anemia in their patients. The absence of superoxide dismutase, a copper enzyme of the erythrocyte, was also noted. This enzyme is necessary for the disposal of the superoxide radical which can damage the erythrocyte membrane.

When the metabolism of copper was examined in patients suffering from Menkes' disease, it was found that levels of copper and ceruloplasmin were low. This revelation caused a greater focus on the fate or role of copper in the disease. Copper deficiency was also seen in the liver and brains of all patients studied. When patients were given copper intravenously, it was incorporated into ceruloplasmin, and other tissue proteins. Oral administration of copper resulted in no significant clinical or biochemical changes.

These observations lead to the conclusion that Menkes' syndrome was due to abnormal uptake of copper by the intestine and that all the characteristics of the disease could be related to this defect (Danks et al., 1972b). However, only seven months later in a follow-up paper, it was reported that the defect was not in the uptake of copper by the duodenal mucosa brush border cells, but in the transport within the cells or the transport across the serosal cell membrane (Danks et al., 1973).

As mentioned earlier, copper was given intravenously (Danks et al., 1972b) in order to by-pass the intestinal defect. Serum copper and ceruloplasmin levels were normalized by intravenous copper therapy in most infants affected by Menkes' syndrome (Danks et al., 1972a, 1972b; Garnica et al., 1974; Grover and Scrutton, 1975; and Danks et al., 1974) which was generally accompanied by some clinical improvements (Danks et al., 1972a; Dekaban and Steusing, 1974; Grover and Scrutton, 1975; and Lott et al., 1975). Parenteral copper therapy was thus recommended as possible treatment (Danks et al., 1972a, 1972b). Intravenous infusions administered slowly over a period of hours (Danks et al., 1973) and intramuscular injections of copper EDTA (Walker-Smith et al., 1973) raised serum copper and ceruloplasmin levels. However, copper therapy, by infusions or injections, did not reverse the neurological deficits of the patients (Dekaban and Steusing, 1974). Intravenous infusion of copper sulfate or copper acetate to two cousins of ages three days and two and one

half months, resulted in some benefit to the younger infant. It was noted that the younger infant at six months of age had reached a functional level of four months, but the older infant showed continual neurological degeneration and died at the age of fifteen months. These results were translated to mean that early treatment could be important in the prevention of neurological and biochemical disfunctions (Grover and Scrutton, 1975). Garnica et al. (1977) suggested that this defect in membrane transport or abnormal transport or storage protein is already clinically important in utero, which could account for the early abnormalities in newborns and the poor clinical response to parenteral copper therapy.

Danks et al. (1973) mentioned that an intestinal copper transport protein similar to the one found in the intestinal cytosol of chicks by Starcher (1969) may be defective or missing. It is known that copper is absorbed in the small intestines, particularly in the duodenal-jejunal region (Bush et al., 1956; Van Campen and Mitchell, 1965). There are three separate mechanisms described for copper absorption, which are (a) passive or simple diffusion of ionic copper (Mills, 1956; Marceau et al., 1970); (b) energy dependent-transport involving a copper amino acid complex to the mucosa of the small intestine (Crampton et al., 1965; Kirchgessner et al., 1970); (c) energy-dependent and endocytotic uptake of copper by the duodenal epithelium (Johnson and Evans, 1980). Evans and Cornatzer (1970) identified a copper and zinc metalloprotein of

molecular weight 10,000 from rat duodenal cytoplasmic fractions. In vitro kenetic studies with ^{64}Cu and ^{65}Zn, showed that orally administered copper and zinc would bind the duodenal protein, but as the amount of duodenal bound ^{64}Cu decreased with time both plasma and liver levels increased. Following absorption ^{64}Cu is first associated with albumin and after several hours is found in plasma ceruloplasmin. In 1976, Evans and LeBlanc isolated and characterized a copper-binding protein from the cytosol of rat intestines. This protein seemed to be different from the conventional metallothionein described by Prins and Van den Hamer (1981). According to Kaji and Nordberg (1979), metallothionein is a cysteine-rich cytoplasmic metal-binding protein. It was suggested that this copper-binding protein plays a role in regulating the passage of copper from the mucosal cells to the blood. Johnson and Evans (1980) had reported that a 9,000 dalton soluble protein from the small intestine of five day old rats separated into two fractions by DEAE sephadex chromatography showed low absorbance at 280 nm, contained a small amount of aromatic amino acid and a large amount of cysteine. All of these properties were consistent with it being a metallothionein. In a very recent report, cultures of lymphoblasts taken from Menkes' and normal cells. It could be deduced from these observations that enhanced ability of mucosal cells from the gastrointestinal tract of Menkes' patients to synthesize metallothionein may account for their increased retention of copper in the small bowel (Riodan and Jolicoeur-Paquet, 1982).

There has been very little information concerning membrane bound copper transport proteins. However, Everett (1981) used sodium dodecyl sulfate-polyacrylamide gel electrophoresis, to show that there was a missing or insufficient quantity of copper transport protein in the membrane of the small intestinal mucosal cells of the male mottled brindle (Mo^{br}) mice, as compared to normal black male (+/o) and heterozygous female mice ($Mo^{br/+}$). This copper-binding protein showed on-off binding kinetics for copper. Binding of copper is competitively and non-competitively inhibited by zinc and cadmium, respectively. At present, characterization of this copper transport protein is far from complete.

A model used in the investigation of Menkes' syndrome is the brindled strain of the mottled mutant mouse (Mo^{br}) Mus musculus. it has an X-linked allele of the mottled locus of the mutant (Mo) and a deficiency in copper transport (Fraser et al., 1953).

Everett (1978) and Yajima and Suzuki (1979) described the general similarities of the neurological mutant mouse compared with those of Menkes' syndrome in humans. The affected male mice have abnormal white hair, altered hair structure (pili torti, monilethrix, trichorrhexis nodosa), retardation in development followed by early death, defect in intestinal copper absorption decreased activity of diamine oxidase, cytochrome oxidase, dopamine-β-hydroxylase, and tyrosinase.

The hemizygous males (Mo^{br}) are characterized by curly whiskers, the absence of hair pigment, progressive inactivity, loss of weight

at about the tenth to twelfth days postnatally and death at about the fourteenth day. The brain weight was less than 75% that of normal littermates.

In earlier in vitro studies, Hunt (1974) demonstrated that the uptake of ^{64}Cu into the intestinal cells from the lumen of the Mo^{br} males was not due to defective absorption into the intestinal epithelium, that is, that the uptake from the lumen is normal. The general body deficiency was due to a defective transport from within the mucosal cells to the blood.

Affected human infants and neonatal mottled mice had copper deficiencies in many tissues, such as the serum (Hunt, 1974), the brain and the liver (Hunt and Port, 1979; Everett, 1978). Copper, nevertheless, accumulates in the kidney as well as in the intestinal mucosal cells.

Horn and Jensen (1980) and Sheedlo and Beck (1981) reported that copper was localized on the surface of the brush border of the duodenal mucosal in Menkes' patients and in heterozygous tortoise shell ($Mo^{to}/+$) female mice, respectively. Both investigations utilized an ultrastructural histochemical copper localization technique for analysis. In the work of Sheedlo and Beck (1981), the copper seemed to accumulate not only along the brush border surface of the duodenum, but also in pinocytotic vesicles at the base of microvilli of the $Mo^{to}/+$ female mice. Normal female (+/+) mice did not show accumulation of copper along the duodenal microvilli. The

results of investigations seem to point to a defect in intestinal copper uptake, as well as to a defect in a copper transport mechanism.

Overall, the literature describes defects in copper transport which underlies the metabolic defects in Menkes' syndrome and in Mo^{br} mice. It is evident that the mechanism(s) of copper absorption from the lumen of the small intestine into the mucosal cells, its intracellular transport to the serosal side of the cells, and across the membranes into the portal blood is not clearly understood. The progression of investigations in this area does seem to indicate optimism to elucidation of a better understanding of the mode of intestinal copper transport.

CHAPTER III

MATERIALS AND METHODS

Materials

Chemicals

All chemicals used, unless otherwise specified, were obtained from Sigma Chemical Company. Electrophoretic chemicals were obtained from Bio-Rad Laboratories. Glacial acetic acid, glycine and ascorbate were obtained from Fisher Chemical Company. Methanol was obtained from Scientific Products. Deionized distilled water was used in all experiments.

Animals

Animals used were from an ongoing laboratory stock. The normal Black Agouti males and mottled brindle (Mo^{br}) female mice were mated. The offsprings of these matings, i.e., normal males, affected males and heterozygous females were used from birth as specified. The mice were housed in plastic cages with cedar bedding. The adults were fed ad libitum on Purina Mouse Chow (15 mg copper/gram weight pellet) and tap water. Pups nursed their natural mothers.

Equipment

The major units of equipment used were the: Beckman J2-21 centrifuge, Beckman Model 3500 pH meter, Beckman Model 35 Spectrophotometer, Beckman DU-8B Spectrophotometer Slab Gel Scan System, Bio-Rad Laboratories Model 220 Dual Slab Electrophoresis

Apparatus, Mettler H54 Analytical Balance, Virtis Freezemobile 12 Lyopholizer, Savant Instruments Model HV 1000 Power Supply Unit, and Scientific Industries Vortex-Genie Mixer Model K-550-C.

Methods of Procedure

Animal Sacrifice and Tissue Removal

Animals used were sacrificed by decapitation, the first 10cm of the small intestines were removed and immediately used for isolation of mucosal cells or homogenation of the whole tissue for membrane isolation. All membrane isolation steps were done on ice or at 0-4C.

Membrane Isolation

Membrane isolation was done according to the method used by Everett (1981). In preparations of mucosal cell membrane, the small intestines were removed and inverted over a glass stirring rod, tied with suture thread at the upper end, rinsed gently with cold 0.9% Manitol-Sucrose-Tris (MST) solution at pH 7.4 to remove food debris. The tissue was then shaken vigorously in a test tube containing more cold MST to remove the mucosal cells. Mixing inverted small intestine segments in cold MST was also an effective means of removing mucosal cells. The isolated cell suspension was then centrifuged at 10,000 rpms for one minute. The pellet which contained whole cells and nuclei was saved and the supernatant was discarded. The pellet was suspended in five volumes of cold water and allowed to stand for no less than 45 minutes or frozen and thawed to disrupt the cell membrane. The solution was centrifuged at 10,000

rpms for three minutes to pellet the membranes. The supernatant contained the cytosol. The membrane pellet was washed several times as in the last step above, until it appeared fluffy white. After the last wash the pellet was resuspended in three volumes of water and frozen. The frozen membrane solution was then lyophilized for 12-18 hours. In order to prepare membrane fractions from the whole small intestinal segment, the tissue was placed in cold MST solution, minced and homogenized by a motor driven pestle at low speed for 10 to 15 seconds. The solution was then rapidly mixed using the mixer and allowed to stand for two minutes. After the tissue debris settled out of solution, the top solution was decanted into centrifuge tubes. The remaining steps followed the procedure outlined above for the mucosal cell membrane isolation.

Membrane Protein Estimation

Membrane protein estimation was done according to the ultraviolet absorption method of Warburg and Christian (1941). The solutions were prepared by diluting the membrane sample in 0.5M Tris-HCl buffer at pH 7.0. The protein standards used were Bovine serum albumin and trypsin.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 10% Slab Gel

The method of Laemmli (1970) in combination with that of Weber and Osborn (1969) as modified by Everett (1981) was used for electrophoretic separation of solubilized intrinsic membrane protein

samples placed in buffer and boiled for two minutes or until samples dissolved. The membrane protein concentration applied to each sample well was 0.6-1.0 mg. After a short run at 50mA to allow the bromophenol blue marker to reach the top of the 10% separating gel, the electrophoresis was continued at 150mA for about 2.5 hours or until the marker was about 0.5 cm from the bottom of the gel.

Staining and Destaining of Slab Gels

Protein staining and destaining were done as described by Weber and Osborn (1969) and spectrophotometric scan analysis was done at 570 nm.

Detection of Copper Complexes

Spectrophotometric scanning was performed at 365 nm and 610 nm. Copper-protein complexes have strong absorbancy at 365 nm, which is indicative of simple copper complexed proteins and the other at 610 nm, which is indicative of copper binding to a high cysteine-containing protein such as seen with metallothionein (Jameson, 1981). The gels were incubated in a copper sulfate solution then scanned at both 365 nm and 610 nm to determine which type of copper-protein complex was present in the position of the desired band.

CHAPTER IV

EXPERIMENTAL RESULTS

Intestinal membrane samples were analyzed by 10% SDS-PAGE. Fig. 1-3 indicates protein profiles of normal membrane samples of homogenized duodenal tissue from 14 day old postnatal mice (tracks D-D_1) and mucosal cells of 28 day old postnatal mice (tracks B-B_2) and Mo^{br} membrane samples of homogenized duodenal tissue from 14 day old postnatal mice (tracks C-C_1). A low molecular weight protein band (B3) was not present in any appreciable quantities in Mo^{br} membrane samples but was present in samples from normal Black Agouti mice. The banding profile of normal 14 and 28 day postnatal intestinal membrane samples showed some differences but band B3 was present in both samples. Mo^{br} male mice also showed three protein bands that were deficient in normal Black Agouti mice. Molecular weight standards (tracks A and E) ranging from 66,000 - 14,300 daltons were electrophoresed with each gel.

The banding profile and grouping is shown in Figure 2 for ease in identification of bands. Bands were grouped alphabetically from A-G, with A having the lowest molecular weight bands and G the highest. The bands in each group were then labeled with Arabic numbers, band 1 having the highest molecular weight. Bands that were present in the Mo^{br} samples, but not in the normal samples, were

Figure 1. 10% SDS-PAGE banding profile of membrane samples and molecular weight standards. A and E: molecular weight standards are indicated on the left; B and B_1: mucosal cell membrane proteins from 28-day-old mice; C and C_1: membrane from homogenized duodenal tissue of 14-day postnatal diseased animals; D and D_1: membrane from homogenized duodenal tissue of 14-day postnatal normal animals. Notice that the low molecular weight band is absent in C and C_1. (Arrows indicate presence in normals.)

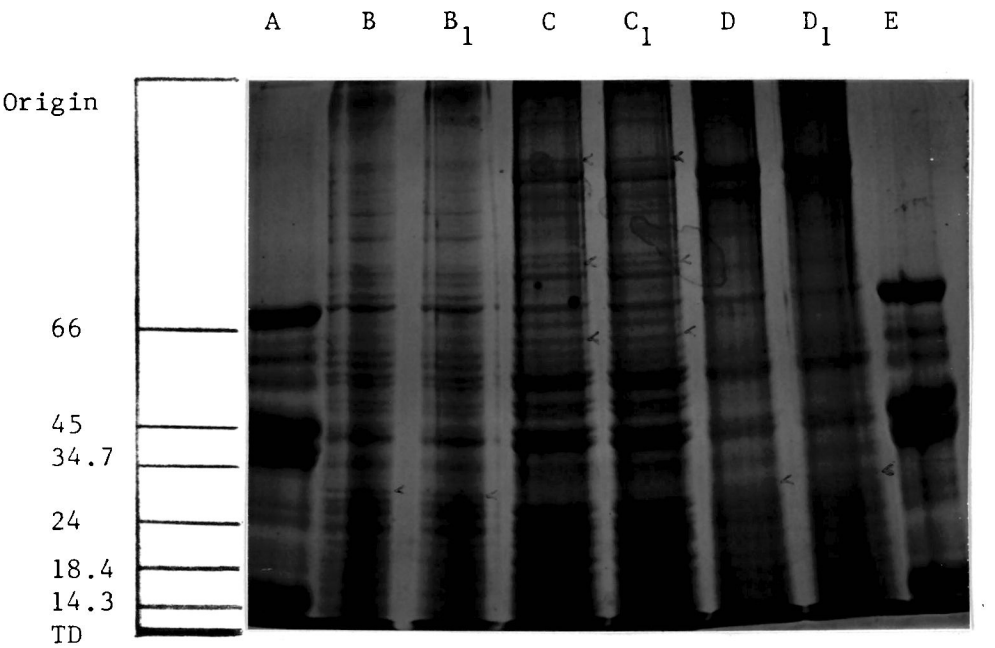

A
B
B1
C
C1
D
D1
E
Origin
66
45
34.7
24
18.4
14.3
TD

Figure 2. Schematic diagram of SDS-PAGE band profile with grouping shown for ease of band identification in Mo^{br} and normal Black Agouti intestimal membrane samples. Molecular weight standards are indicated on the left. Intestinal membrane samples of 14-day postnatal mice were prepared by homogenization of the whole duodenal segment in cold 0.9% Mannitol. Sucrose, Tris buffer (pH = 7.4) then treated with 1% SDS and 5% -Mercaptoethanol.

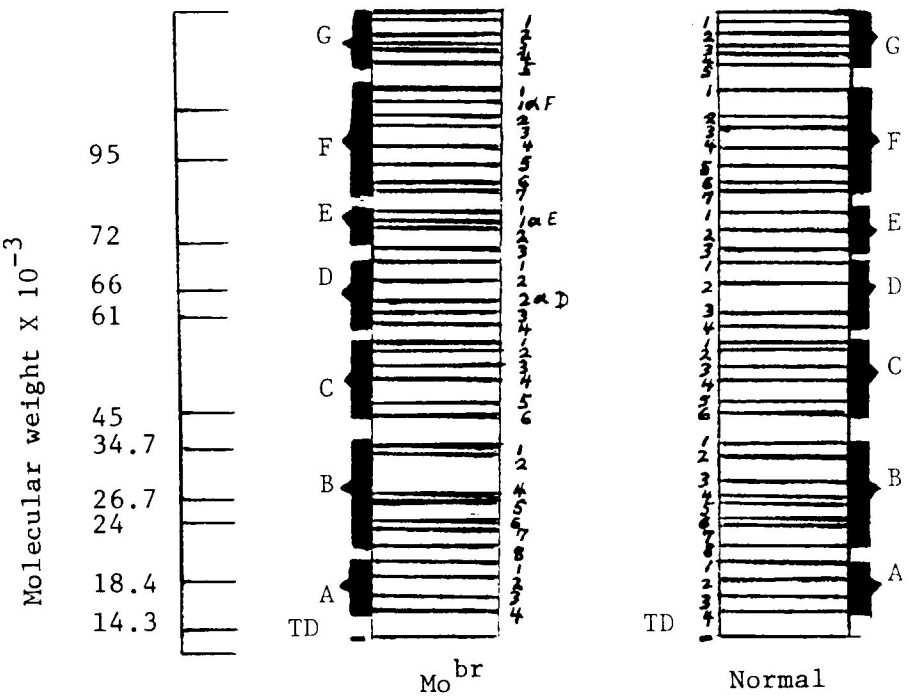
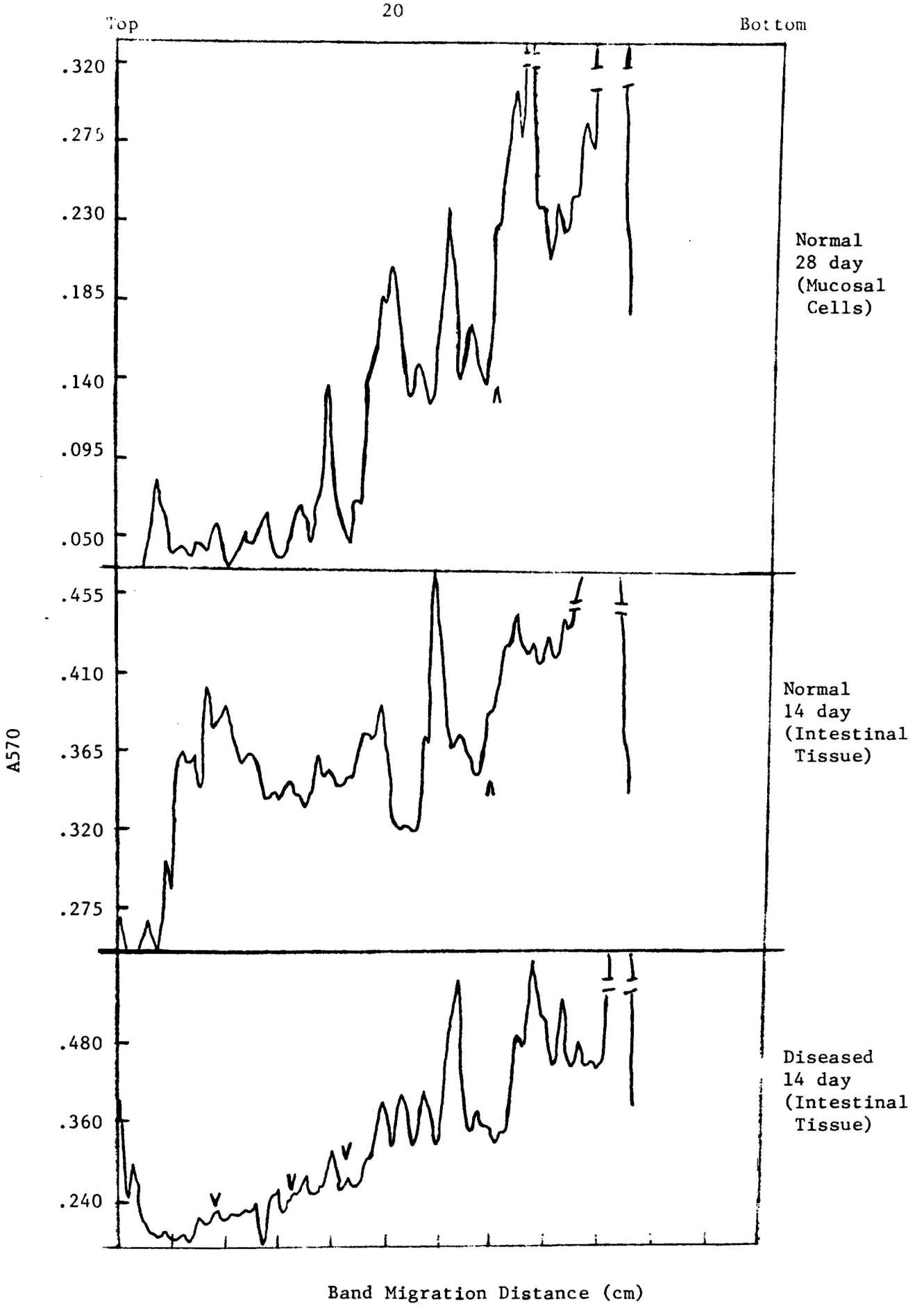

Molecular weight X 10^{-3}
95
72
66
61
45
34.7
26.7
24
18.4
14.3
G
F
E
D
C
B
A
TD
Mo br
Normal

Figure 3. Spectrophotometric scan profiles of coomassie brilliant blue stained polyacrylamide gel in Figure 1. Normal 28 day mucosal cell membrane, normal 14 day and diseased 14 day homogenized intestinal tissue are represented by tracks B, D_1 and C, respectively, in Figure 1. Band B3 is indicated by arrows ( ) in normal samples. Bands F1 , E1 and D2 are indicated by arrows (V) respectively from left to right in the diseased sample.


Top
Bottom
.320
.275
.230
.185
.140
.095
.050
Normal
28 day
(Mucosal
Cells)
.455
.410
.365
.320
.275
Normal
14 day
(Intestinal
Tissue)
A570
.480
.360
.240
Diseased
14 day
(Intestinal
Tissue)
Band Migration Distance (cm)

labeled with the numbers of the last band common to both sample groups and designated with a Greek letter, i.e., α (alpha).

Table I summarizes the major band differences in normal Black Agouti and Mo^{br} male mice at 14 days postnatally.

Figure 3 shows the spectrophotometric scan profiles of the Coomassie Brilliant Blue stained polyacrylamide gel in Figure 1. The scans showed the migration of band B3 in membrane samples from normal Black Agouti mice duodenal mucosal cells of 28 day old and homogenized duogenal tissue of 14 day old (tracks B and D of Figure 1, respectively). The scan also showed the migration of bands F1α, E1α, and D2α from homogenized duodenal tissue of 14 day old Mo^{br} mice (track C in Figure 1).

Figure 4 shows the average molecular weight estimations of standards and of protein bands B3, D2α, E1α and F1α. Molecular weight values of standard proteins versus RF (relative mobility of the tracking dye) showed a linear relation. Molecular weight estimations of unknown bands of samples were then extrapolated from the plot as a function of the abscissa (RF) intercept with the line and its relation to the ordinate (molecular weight).

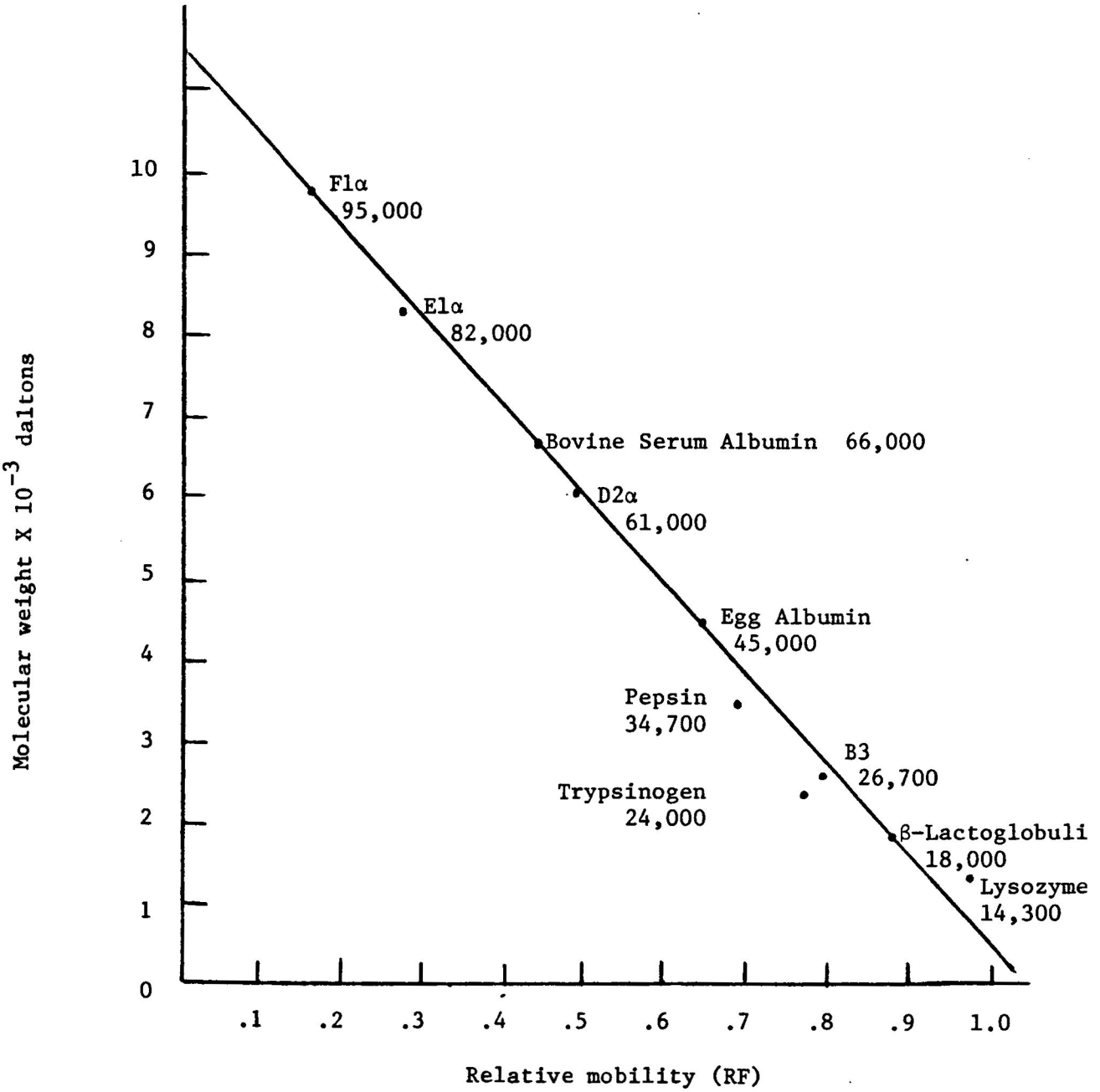
$$\text{Relative Mobility} = RF = \frac{\text{Sample (mm) - origin (mm)}}{\text{Reference (Tracking dye) - origin (mm)}}$$

The average molecular weight estimations calculated from six different experiments for bands B3, D2α, E1α and F1α were approximately 26,700, 61,000, 82,000 and 95,000, respectively.

Table 1. Major band differences in normal Black Agouti and Mo^{br} male mice at age 14 day postnatal. + = present, - = absent, (-) = barely present.

Band	B3	D2α	E1α	F1α
Normal ♂	+	(-)?	-	-
Mo^{br} ♂	-	+	+	+

Figure 4. Average molecular weight estimation of standards and of protein bands B3, D2α, Elα and Flα.


Molecular weight X 10^{-3} daltons
10
9
8
7
6
5
4
3
2
1
0
Flα
95,000
Elα
82,000
Bovine Serum Albumin 66,000
D2α
61,000
Egg Albumin
45,000
Pepsin
34,700
Trypsinogen
24,000
B3
26,700
β-Lactoglobuli
18,000
Lysozyme
14,300
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Relative mobility (RF)

In Figures 5-8 the development of band B3 in normal Black Agouti was monitored by spectrophotometric gel scans of small intestinal membrane samples at varying postnatal ages as indicated by its copper binding in the presence of $CuSO_4$ (Figures 5-6) at 365 nm and in the presence and absence of $CuSO_4$ (Figures 7-8) at 610 nm. At 365 nm a gradual increase was seen from day 1 to day 13. However, at wavelength 610 nm there was a decrease in band B3 from day 0 to day 1 with a sharp increase until day 7 and decreasing sharply by day 14.

Figure 5. Spectrophotometric gel scan profile of normal Black Agouti small intestinal membrane samples at 365 nm in the presence of $CuSO_4$. Arrows ( ) indicate band B3.

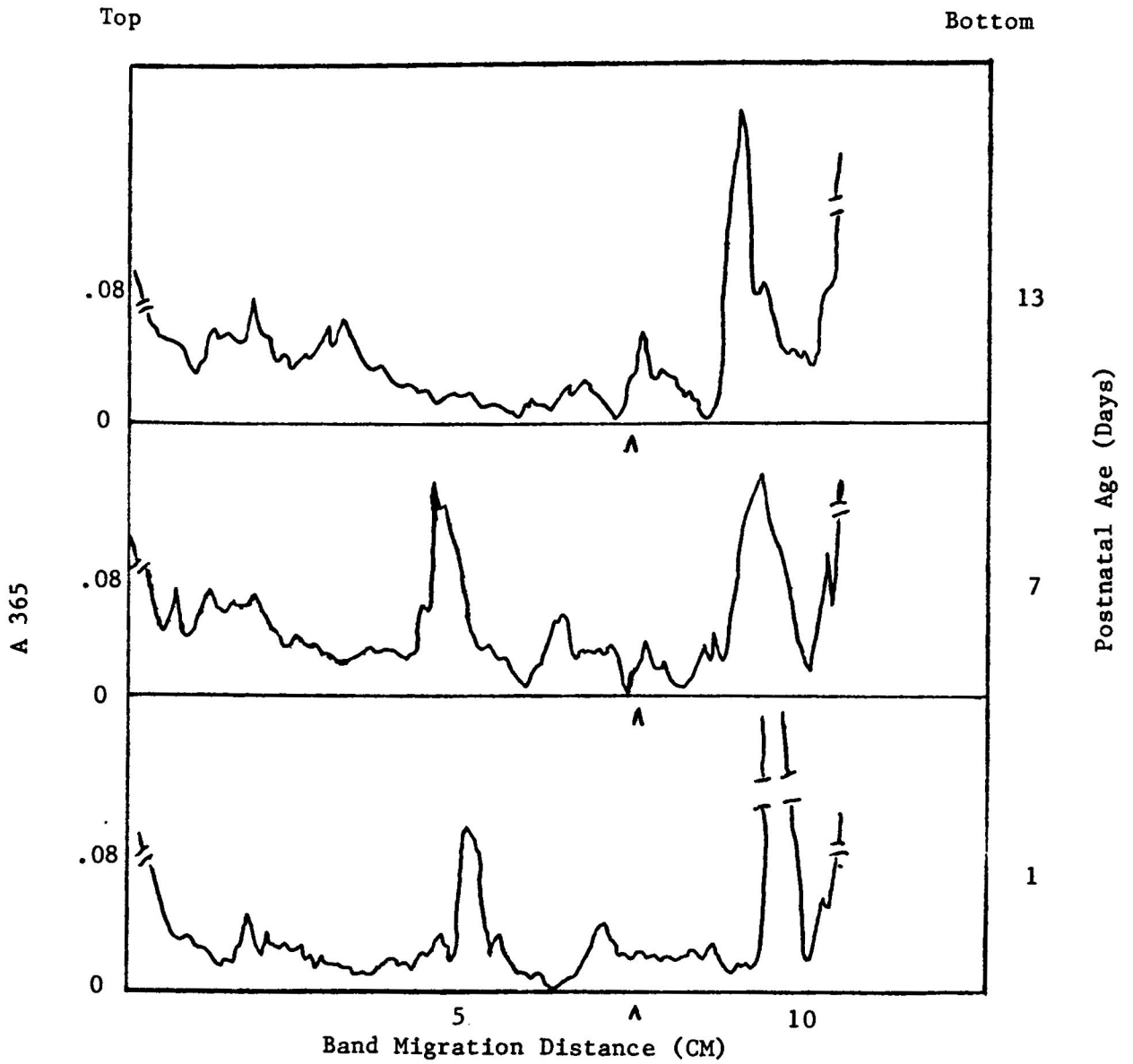

Top
Bottom
.08
0
13
.08
0
7
.08
0
1
A 365
Postnatal Age (Days)
5
10
Band Migration Distance (CM)

Figure 6. Normal development of copper binding protein (Band B3) in Black Agouti mice, showing a gradual increase from day 1 to day 13 when scanned at 365 nm.

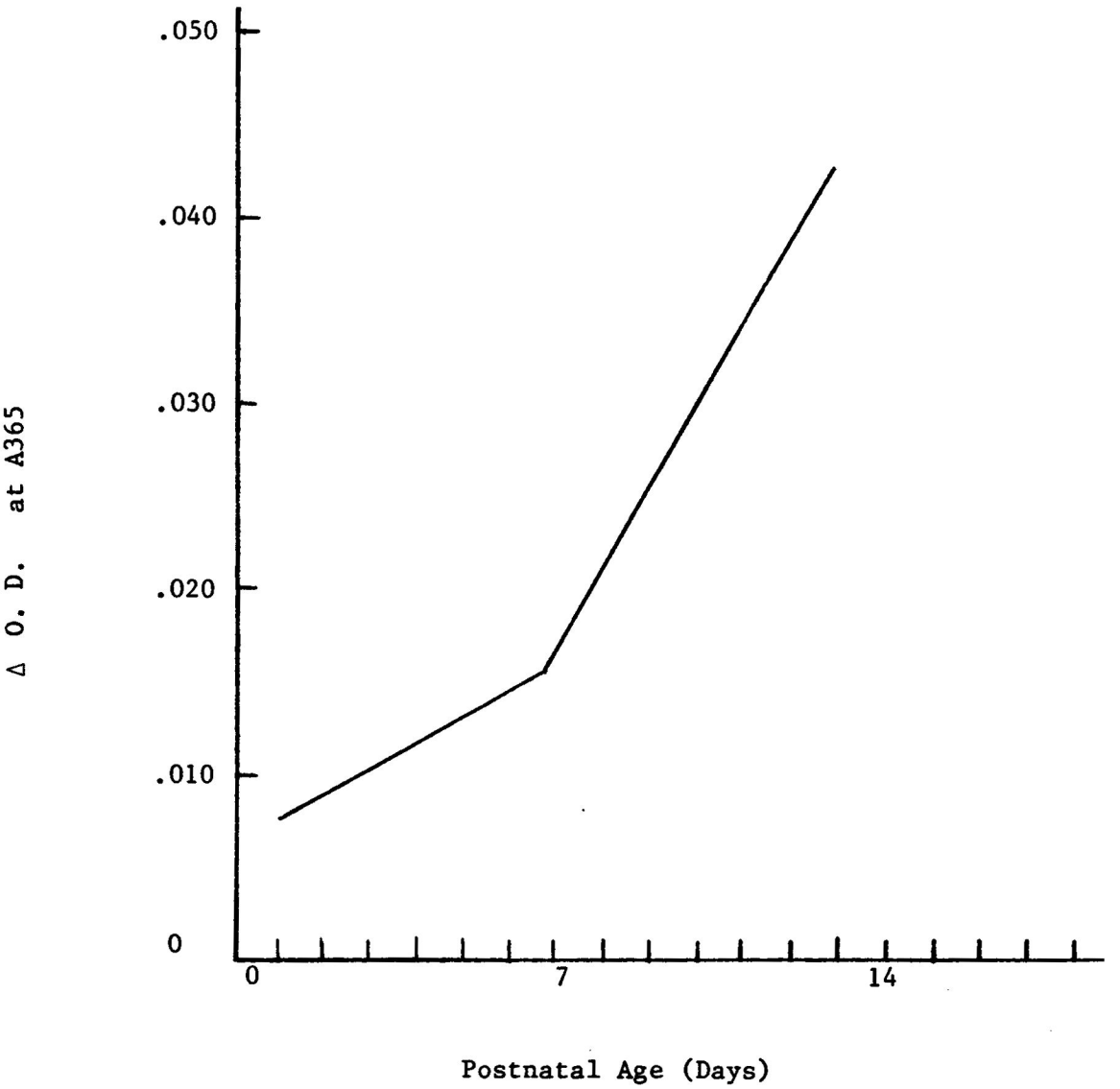

.050
.040
.030
.020
.010
0
Δ O. D. at A365
0
7
14
Postnatal Age (Days)

Figure 7. Spectrophotometric Gel Scan Profile (λ= 610 nm) of Normal Black Agouti samples without copper sulfate added (-) and following incubation of gel in copper sulfate solution (----). Arrows ( ) indicate band (B3).

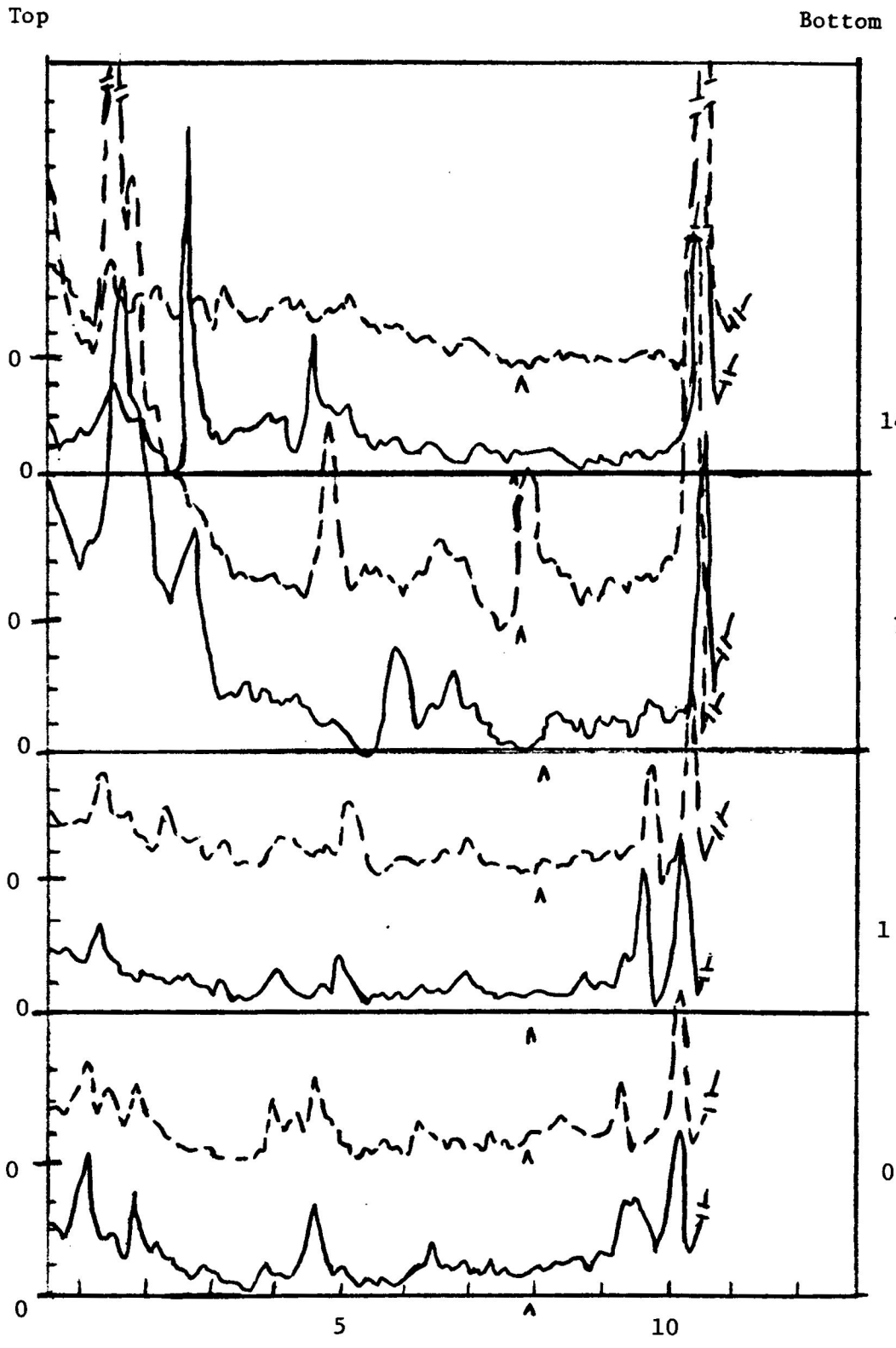
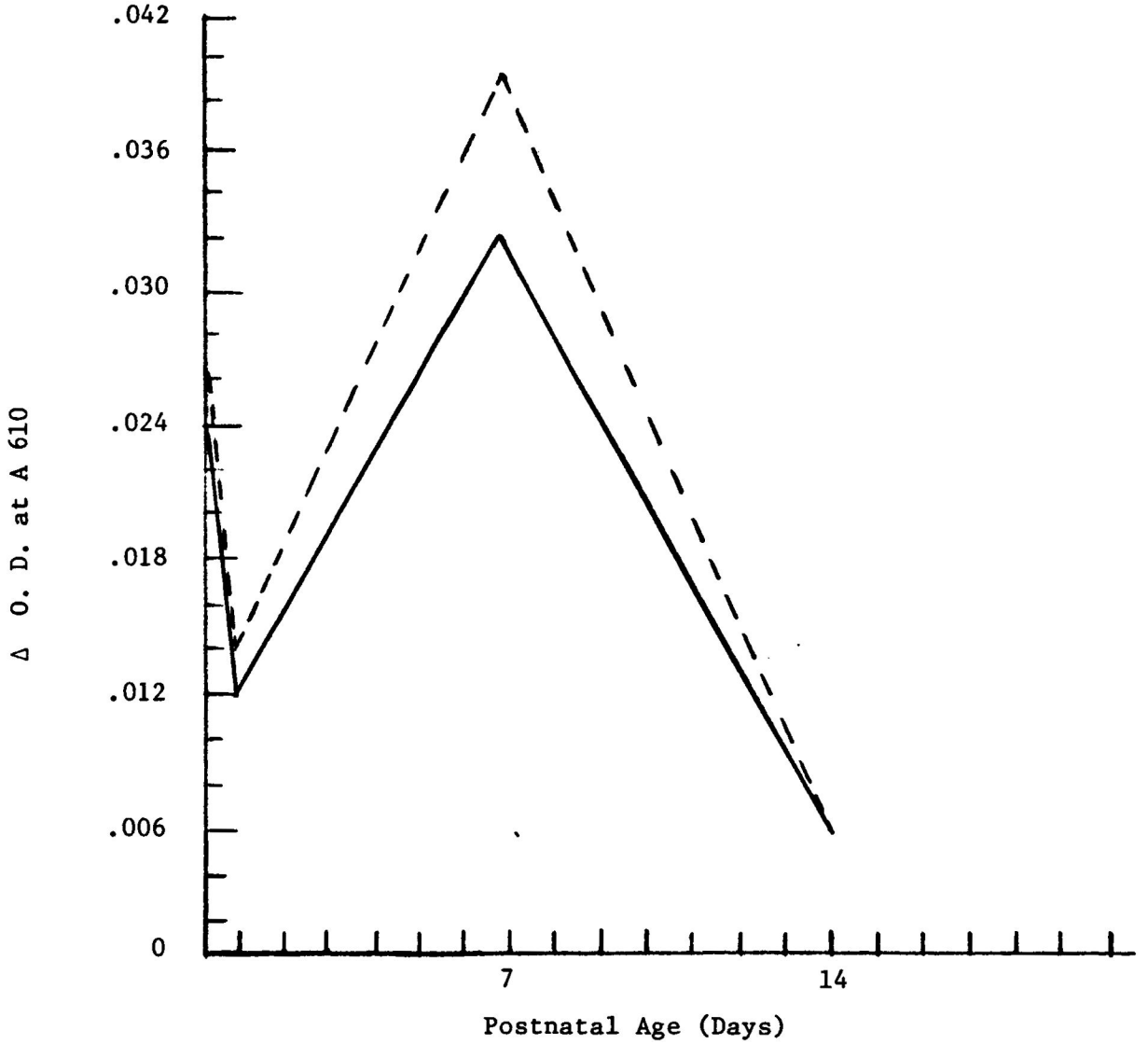

Top
Bottom
0
0
14
0
0
7
0
0
1
0
0
0
0
5
10
Band Migration Distance (cm)
Postnatal Age (Days)
A610

Figure 8. Plot of Figure 7 monitoring the normal postnatal development of Band B3 in Black Agouti mice, showing changes from day 0 to day 14 assayed in the presence (---) and absence (---) of copper sulfate. The plot indicates the average of 3 different experiments at 610 nm.


.042
.036
.030
.024
.018
.012
.006
0
Δ O. D. at A 610
7
14
Postnatal Age (Days)

CHAPTER V

DISCUSSION

Visual and spectrophotometric comparison of SDS-PAGE band profiles of intrinsic intestinal membrane proteins from normal and Mo^{br} male mice revealed that band B3 was deficient in the diseased animal. Band B3 may not be totally absent in the diseased animal, but it is undoubtably not present in adequate quantities to subserve its normal function. Close observation also revealed the appearance of bands D2α, E1α and F1α in the diseased Mo^{br} samples that were not present in normal membrane samples. The appearance of bands D2α, E1α and F1α in the diseased Mo^{br} intestinal membrane samples is not clear yet. One could probably speculate that these proteins are precursors of band B3. This would suggest that band B3 was possibly synthesized as a non-functional polymeric protein-dimer (D2α), trimer (E1α) and/or tetramer (F1α) - as indicated by their respective molecular weights. However, it could possibly be processed posttranslationally into a multisubunit protein for its protection against attack by digestive enzymes in transport of the protein to its intrinsic membrane location or during its storage. If this were the case, then there is probably a defect at the transcriptional level which would lead to a defective polymeric protein or of an enzyme(s) responsible for cleavage of the precursor protein into its functional unit. These possibilities have not yet been tested.

Band D2α stains very faintly in normal Black Agouti membrane samples as seen in the SDS-PAGE profile. This indicates that it is not totally absent but is in very low quantity as compared to the Mo^{br} membrane samples. The fact that band B3 increases in normal mice as band D2α decreases (or disappears) and bands E1α and F1α disappear, lends support to the putative existence of a polymeric protein.

Previous studies in our laboratories by Everett (1981) first recognized this protein (band B3) as being deficient in Mo^{br} males and found it to have certain copper binding activities which should be inherent in a membrane transport protein, i.e., on-off binding kinetics (reversible binding affinities) and the ability of the cations (Zn^{+2} and Cd^{+2}) to inhibit its binding to copper. However, in this work kinetic studies were not done. Certain copper protein complexes were evaluated by scanning the gels at specific wavelength of the visible spectrum. Copper complexes absorbed strongly at (a) 365 nm and (b) type 1 copper proteins involving Cu^{II}, i.e., blue copper proteins with one or more Cu-cysteine bonds, absorbed very intensely at 610 nm (Jameson, 1981). The absorption at 365 nm by band B3 indicated the degree to which copper complexes were formed, and the absorption of band B3 at 610 nm indicates the presence of type 1 Cu^{II}-protein.

In early postnatal development of mottled brindle mice there seems to be a gradual development of symptoms related to copper

deficiency. Similar changes have been indicated in genetic defects in connective tissue or in nutritional copper deficiency of animals (Marston, 1952; O'Dell et al., 1961; Gillespie, 1964; Carnes, 1971; and Danks et al., 1972b). Experiments to monitor the neonatal development of band B3 (copper binding protein) seems to parallel the animals' increasing need for dietary copper. At wavelength 365 nm there seemed to be a gradual increase of copper binding protein seen from day 1 to day 13, but at wavelength 610 nm there was a high quantity of band B3 present at birth (day 0) which decreased by day 1 then increased by day 7 to a maximum level and decreased until day 14. The level of band B3 was not observed at 365 nm at day 0, therefore, no correlation could be made to day 0 at 610 nm. The results observed beyond day 7 seemed contradictory at the two isomeric forms of band B3. When one form of band B3 was decreasing (as seen at 610 nm), the other isomer form was approaching its maximum level (as seen at 365 nm). However, isomeric forms of band B3 have not yet been isolated. The fact that the Mo^{br} males gradually develop symptoms related to copper deficiency as a function of age, and begin a rapid deterioration at about 10-12 days postnatally until the animal dies at about 14-16 days (Hunt, 1974), is supported by the gradual increase seen from day 1 to day 13 and by the decrease seen from day 7 to day 14 in normal mice.

Spectrophotometric gel scan profiles of normal intestinal membrane samples taken from Black Agouti mice also revealed that

there were dynamic changes in the intrinsic proteins during postnatal development. These profiles seem to stabilize to some degree at about two weeks, as seen in comparison of SDS-PAGE profiles of normal samples taken from two week and four week male mice in Figure 1. Based on this data and the data presented in Figure 8 at day 0 showing band B in high quantity it could be assumed that there is a possibility of a fetal or isomeric form of the copper binding protein which changes with postnatal development into new or adult forms of the protein. An example of such a postnatal developmental change is seen in the case of fetal hemoglobin which is replaced with age by another isomeric form, adult hemoglobin in humans. The diseased animals are able to survive the early postnatal period, primarily because of the presence of copper derived from placental transport. Since the putative fetal copper transport protein is not present in the Mo^{br} diseased animal, it will die unless parental copper is administered before the fatal decrease in _in utero_ acquired copper.

Copper therapy (like available copper in the normal) can secondarily lead to the induction of the adult form of the copper transport protein. Precedence for such a mechanism does exist for apoferritin synthesized but which does not leave polyribosomes until Fe^{III} is bound to the apoprotein. The presence of apoferritin also inhibits synthesis of the protein.

If such a mechanism does exist for the transport of copper this will create a vicious cycle which can be broken by making copper available parenterally.

CHAPTER VI

SUMMARY

We find our work and data significant in that knowledge of this newly identified intrinsic membrane copper binding protein is very limited. Many investigators have reported cytosolic copper binding proteins (Starcher, 1969; Evans and LeBlanc, 1976) but our finding of a membrane bound protein associated with copper transport is unique in this sense. Thus, any information contributing to knowledge of the characterization, development and/or function of this protein is important in helping to increase the understanding of the mechanisms of copper transport. It is evident that the mechanism(s) of copper absorption from the lumen of the small intestine into the mucosal cells, its intracellular transport to the serosal side of the cells, and across the membrane into the portal blood is not clearly understood.

In summary:

1) Using 10% SDS-PAGE, a low molecular intrinsic protein of approximately 26,700 daltons (band B3) detected in the small intestine of normal Black Agouti mice was found to be deficient in Mo^{br} male mice.
2) Three protein bands D2α, E1α and F1α of approximately 61,000, 82,000 and 95,000 daltons, respectively, were

present in Mo^{br} male mice but deficient in normal Black Agouti littermates.

3) Developmental studies of band B3 revealed a gradual increase in the amount of copper bound as age increased postnatally, which seems to correspond to an increased need for dietary copper in neonatal development.

4) Spectrophotometric scan profiles indicated dynamic changes in intrinsic membrane proteins with increased postnatal age up to about two weeks.

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